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PRINCIPAL INVESTIGATOR: Meredith McCracken

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**13. ABSTRACT** ***(Maximum 200 Words)***

Protein kinase C (PKC) promotes cell survival in response to ionizing radiation in a variety of experimental models including human carcinoma, human glioblastoma, and transformed mouse embryo fibroblast cell lines. The purpose of this project is to enhance radiation-induced mammary tumor cell death by inhibition of specific PKC isoforms. Antisense oligonucleotides have been introduced into human breast tumor cell lines to selectively inhibit PKC isoforms. MDA-MB-231 and MCF-7 cells treated with oligonucleotides directed against PKC isoforms δ and ζ exhibited impaired survival in response to 5.6Gy gamma radiation as measured by mitochondrial metabolism of tetrazolium dye. PKC δ and ζ oligonucleotides were shown to selectively deplete PKC δ and ζ proteins from cell extracts. Furthermore, a selective PKC δ inhibitor, rottlerin, impaired survival of cells treated with a therapeutically relevant dose of radiation (1.5Gy). Treatment of MDA-MB-231 cells prior to low dose radiation exposure with PKC δ oligonucleotide significantly impaired cell survival. The conclusion of this work is that PKC δ and PKC ζ act as a pro-survival factors in human breast tumor cells *in vitro*. These findings suggest that down-regulation of PKC δ and PKC ζ may be a useful approach to radiosensitization in mammary tumors.

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## Table of Contents

**Introduction:**

The protein kinase C (PKC) family of serine/threonine mediate intracellular responses to a variety of stimuli, including growth factors, hormones and neurotransmitters. PKC participates in abnormal growth processes such as carcinogenesis, tumor progression and inflammation (Goekjian and Jirousek, 1999). The PKC family is composed of twelve members that are divided into three sub-families: classical ($\alpha$, $\beta$I, $\beta$II and $\lambda$), novel ($\delta$, $\varepsilon$, $\eta$, $\theta$ and $\mu$), and atypical ($\zeta$ and $\iota/\gamma$) isoforms. PKC overexpression and increased PKC activity have been observed in human breast cancers compared with normal mammary tissue (O'Brian et al.,1989; Gordge et al., 1996). Furthermore, there is a correlation between elevated PKC protein levels and aggressive breast cancer phenotypes, such as those that lack estrogen receptors and exhibit multidrug resistance (Borner et al., 1987; Lee et al., 1992). PKC gene and protein expression are induced following ionizing radiation (Woloschak et al., 1990; Kim et al., 1992). Inhibition of PKC radiosensitizes human squamous carcinoma, human colon adenocarcinoma, transformed mouse embryo fibroblast, and human glioblastoma cell lines (Hallahan et al., 1992; Zaugg et al., 2001; Rocha et al., 2000; Begemann et al., 1998). The specific role of PKC in radiation response and the individual isoforms involved are not well defined however, the use of PKC inhibitors, such as staurosporine, sangivamycin and H7 have provided evidence that PKC mediates radiation resistance (Begemman et al., 1998; Hallahan et al., 1992; Zhang et al., 1993). We have applied antisense oligonucleotide technology to explore isoform specific functions of PKC in the radiation survival of human mammary carcinoma cell lines *in vitro*. The goal of this project is to enhance radiation-induced mammary tumor cell death with PKC isoform selective inhibitors.

**Body:**

As stated in the July 2000 annual progress report, optimization of antisense oligonucleotide delivery to human mammary tumor cell lines was completed (Task #1). Task #2 proposed that cells which were co-transfected with PKC oligonucleotide and FITC conjugated oligonucleotide be sorted by flow cytometry to enrich for oligonucleotide containing cells. However, for reasons addressed in the accepted July 2000 progress report, this task was abandoned and flow sorting will not be used in any of the future experiments.

Modification of Task #3 from Northern to Western analysis of PKC oligonucleotide treated cells was accepted in the previous progress report. Quantification of PKC α protein reduction in MDA-MB-231 cells and PKC ζ protein reduction MCF-7 and MDA-MB-231 cells by their respective PKC isoform selective oligonucleotide inhibitiors was also previously reported. However, experiments showing reduction of PKC α protein in MCF-7 cells by PKC α oligonucleotide were not successful due to the very low immunodectable levels of PKC α in MCF-7 cells (see page 28, Fig.2 of appended manuscript). During months 13-18, Western blot analysis of protein extracts from PKC δ oligonucleotide treated MCF-7 and MDA-MB-231 cells was performed (Fig. 1). A detailed summary of the results for these experiments may be found on page 12-13 of the appended manuscript in preparation. In short, PKC δ oligonucleotide significantly reduced PKC δ protein levels. PKC δ oligonucleotide was selective for the PKC δ isoform as no reduction in PKC ε, PKC ζ or bcl-2 was observed following treatment with the PKC δ oligonucleotide. The PI concluded that antisense oligonucleotides are efficient and highly selective inhibitors of PKC isoforms. Task #3 is complete.

The partial completion of Task #4 was reported in the first annual summary and concluded that of the five PKC olignucleotides screened, PKC δ and PKC ζ olignucleotides significantly reduced MTS metabolism, used as an index of cell survival, in MDA-MB-231 and MCF-7 cells treated with 5.6Gy radiation (refer to page 29, Fig.3 of appended manuscript). The role of PKC δ in breast tumor cells was of particular interest because contradictory reports exist in the literature regarding the role of PKC δ in cell survival and apoptosis. As an alternative approach to PKC δ isoform selective inhibition at the mRNA level by antisense oligonucleotides, the effect of a site selective PKC δ enzyme inhibitor, rottlerin, was tested (Fig. 2). The survival of cells receiving no radiation, or either 1.5 or 5.2Gy radiation was reduced by rottlerin. A detailed description of these results is reported on page 13-14 of the appended manuscript. The final approach to isoform selective PKC inhibition will use dominant negative plasmids which express kinase dead mutants of PKC α, PKC δ and PKC ζ. The PI has obtained these constructs and will test them in months 26-30. The use of rottlerin and PKC dominant negative constructs was added to Task #4 in the July 2000 report. Task #4 will be complete, following the dominant negative experiments.

Task #5 outlined the optimization of clonogenic survival and MTS assays, with the most effective of these two assays being used as the end-point for survival of PKC oligonucleotide and radiation treated cells. As explained in the previous years progress report, the MTS assay is a rapid and reproducible assay for determination of relative cell survival. Radiation survival curves for MCF-7 and MDA-MB-231 cells were similar (Fig. 3). In MCF-7 cells, MTS and clonogenic survival assays were compared for end-point analyses. Clonogenic survival by the crystal violet staining method was not a suitable end-point for MDA-MB-231 cell survival because these cells fail to form discrete colonies. Results for these experiments are summarized in the appended manuscript (page 10).

Therapeutically, radiation is administered in the range of 1-2Gy/treatment. *In vitro*, 1.5Gy radiation treatment of MCF-7 and MDA-MB-231 cells reduced survival by 20% as measured by

the MTS assay. The PI has also tested the effect of PKC oligonucleotides on the survival of MCF-7 and MDA-MB-231 cells treated with a therapeutic dose of radiation (1.5Gy). When MCF-7 cells were pre-treated with PKC $\alpha$, PKC $\delta$ or PKC $\zeta$ oligonucleotides, there was no significant decrease in cell survival (Fig. 4A) compared to treatment with scrambled oligonucleotide controls. In addition, the clonogenic survival of 1.5Gy irradiated MCF-7 cells was not impaired by pre-treatment with PKC $\delta$ oligonucleotide (data not shown). However, in non-irradiatied and 1.5Gy irrradiated MDA-MB-231 cells pre-treated with PKC $\delta$ oligonucleotide, cell survival was significantly reduced (Fig. 4B). While pre-treatment of 1.5Gy irradiated MCF-7 and MDA-MB-231 cells with PKC $\zeta$ oligonucleotide showed impaired survival compared to the scrambled oligonucleotide, this difference was not statistically significant. Task #5 is complete.

Task #6 proposed to measure radiation-induced DNA damage. The approved modification of this task replaced pulse-field gel electrophoresis with the comet assay to detect DNA damage. The PI's laboratory received a grant for an apoptosis analysis facility, participated in a demonstration of the comet assay system and plans to acquire the instrumentation by September, 2001. During months 28-36, the comet assay will be optimized and experiments performed to test whether PKC isoform selective inhibition attenuates repair of radiation-induced DNA damage. Task #6 is being addressed and should be in progress by October, 2001.

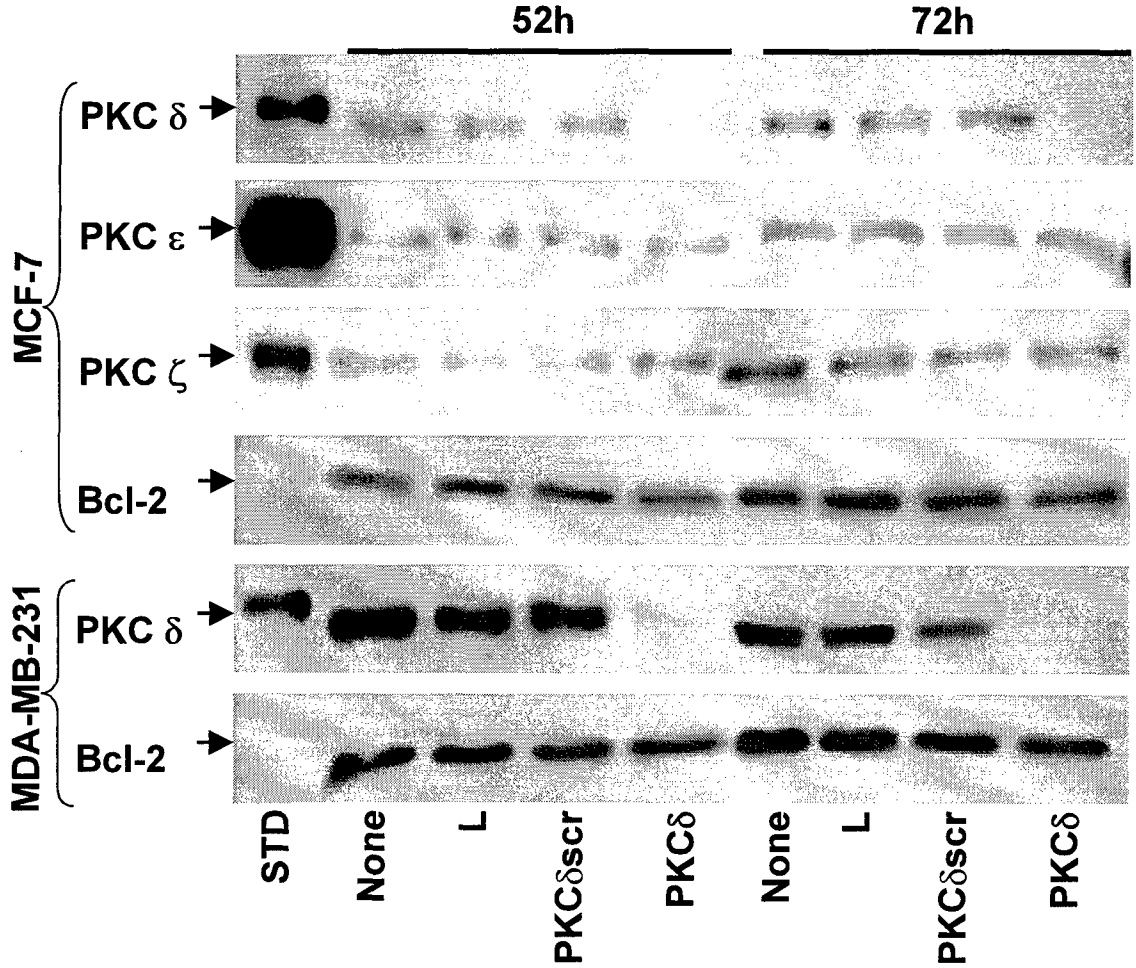



**Fig.1A. Immunoblot analysis of PKC δ oligonucleotide treated cells.** Extracts were prepared from cells 52-72 h after treatment with lipofectin alone (L), 100nM PKC δ oligonucleotide (PKC δ), the nucleotide scrambled version (PKC δ scr), or no treatment. Proteins 20μg/lane were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC δ, PKC ε, PKC ζ, and bcl-2. The PKC standards (STD) included purified PKC δ (15ng/lane), PKC ε (5ng/lane), and PKC ζ (25ng/lane). Results are typical of 3 independent experiments.

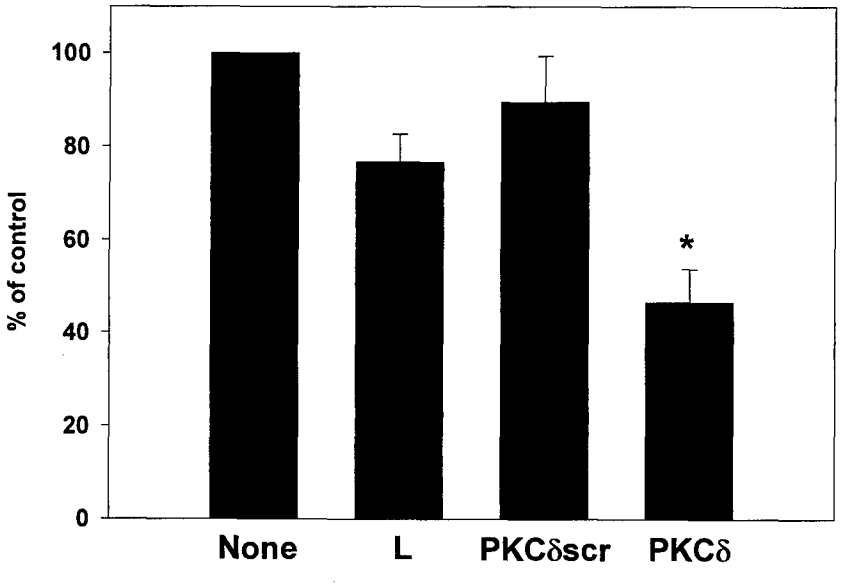



**Fig.1B. Quantification of PKC δ protein in PKC δ oligonucleotide treated MCF-7 cells.** Data are the mean of n=3±SE independent experiments for MCF-7 cell extracts prepared 52 h after treatment as in A. PKC δ signal density was determined by densitometry and normalized to Coomassie stained bands on acrylamide gel with control signal denstity set =100%. Statistically significant difference between PKC δ and PKC δ scrambled oligonucleotide treated cells was determined using the Student t-test and is indicated (*P<0.05).

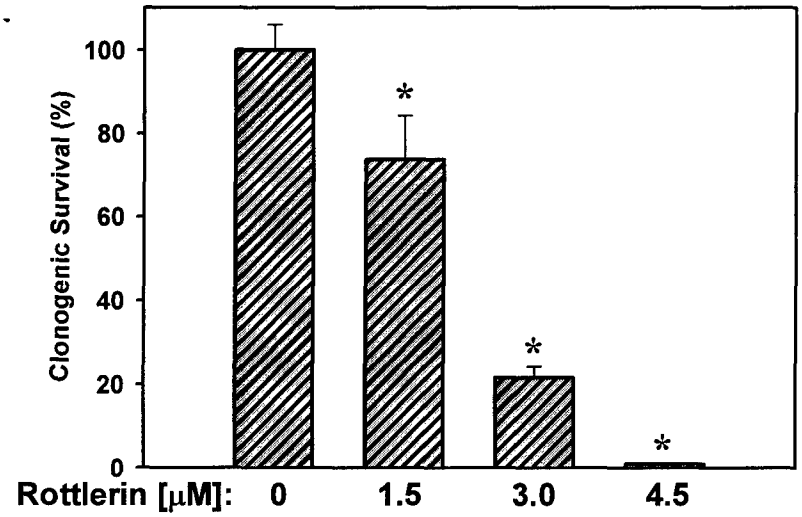
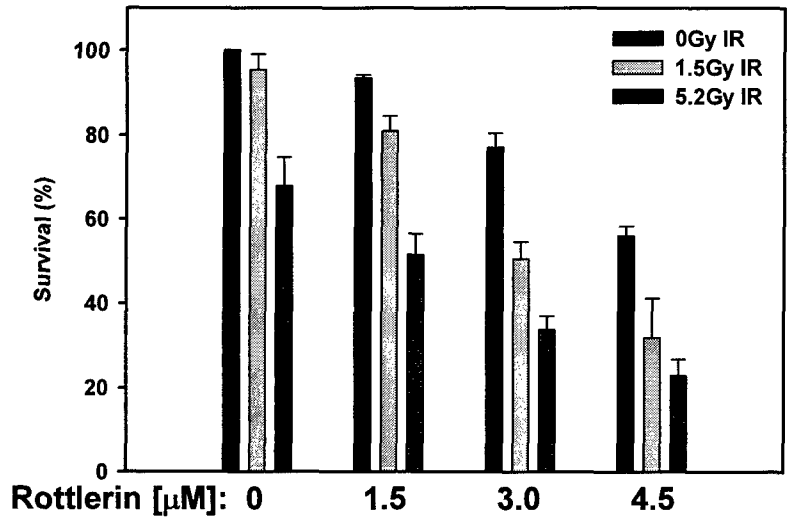
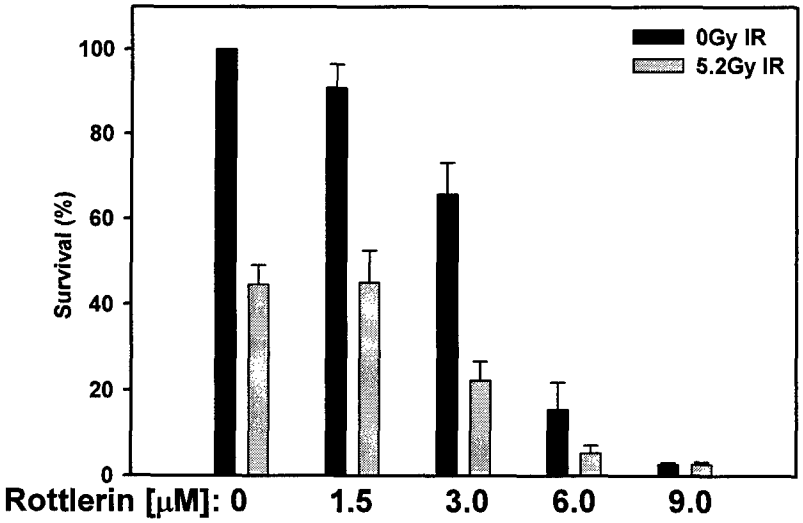



**Fig. 2. Effect of rottlerin on cell survival.** A, MCF-7 cells ($1.0x10^4$/100mm$^2$ dish) were treated with indicated concentrations of rottlerin. Cells were left undisturbed for 7 days then plates were stained with crystal violet to visualize colonies. The cloning efficiency for control cells was 11.0%. The clonogenic survival was determined for n=3±SE independent experiments with control clonogenic survival set =100%. Statistically significant differences between control and treatment groups were determined by one-way ANOVA with Tukey's Multiple Comparison Test and are indicated (*P<0.05).




B, MCF-7 cells ($3.0x10^5$/35mm$^2$ dish) were treated with 1.5 or 5.2Gy IR (radiation), harvested, replated at a cloning cell density of 2500 cells/well, and treated with indicated concentrations of rottlerin. Cells were grown for 7 days and cell survival was determined by MTS assay.




C, MDA-MB-231 cells ($2.0x10^5$/35mm$^2$ dish) were treated as in B but with 5.2Gy IR, replated at a cloning density of 250 cells/well, and grown for 5 days. B and C, Data are the mean of n=3±SE independent experiments performed with 5 replicates/treatment. Cell survival of control cells was set =100%.

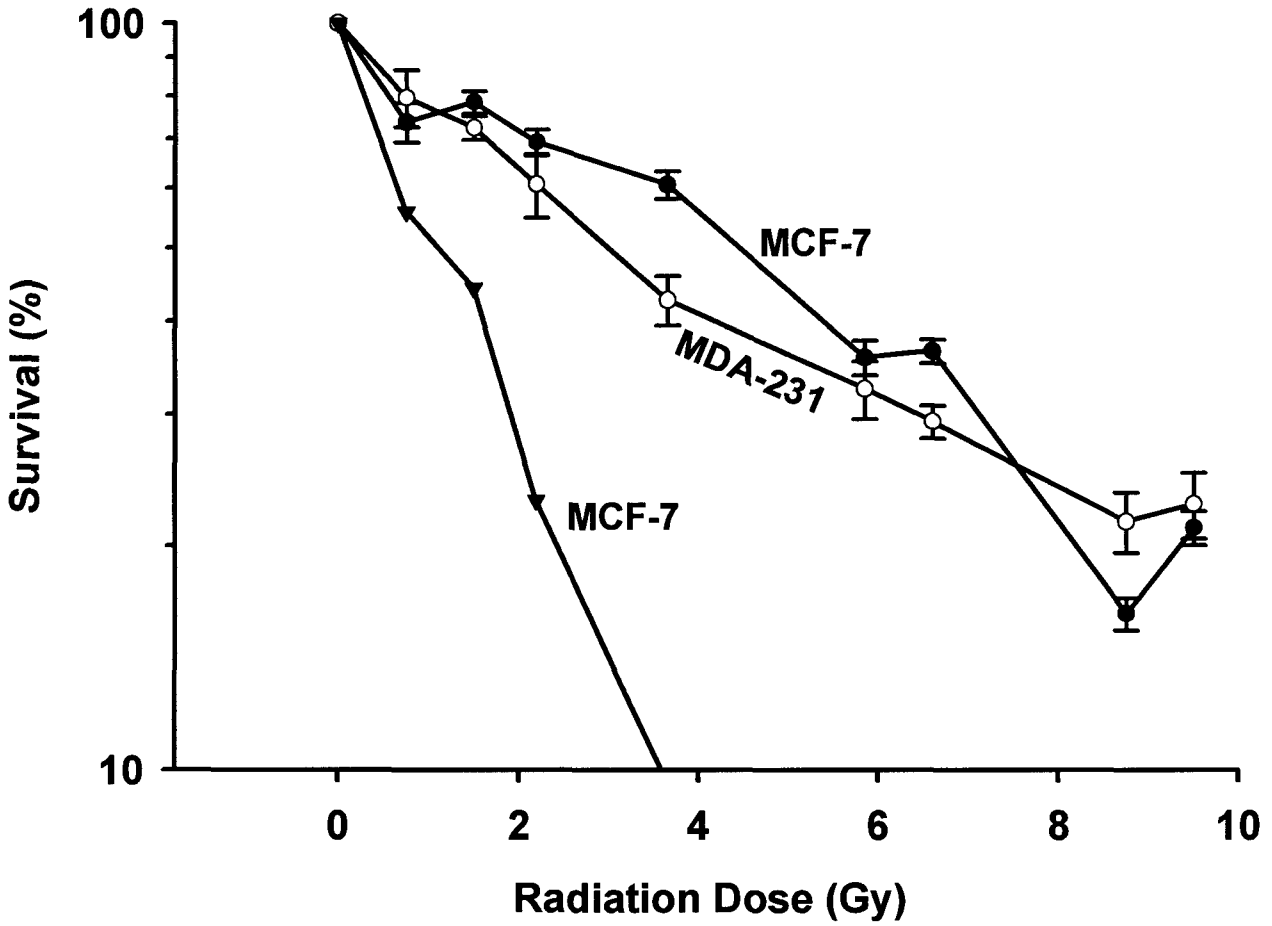



**Fig. 3. Radiation survival curves of MCF-7 and MDA-MB-231 cell lines.** Cells were treated with 0-9.5Gy doses of radiation (IR). Following IR exposure (20h), cells were harvested and replated at cloning cell densities in 96-well plates or 100mm$^2$ dishes. After 7 or 5 days of incubation cell survival was determined by MTS (circles) and clonogenic survival (triangles) assays. Survival of control cells was set =100%. The efficiency of clonogenic survival in control cells was 11.0%. Data are the mean of n=3±SE (MTS data) or n=1 (clonogenic survival) independent experiments.

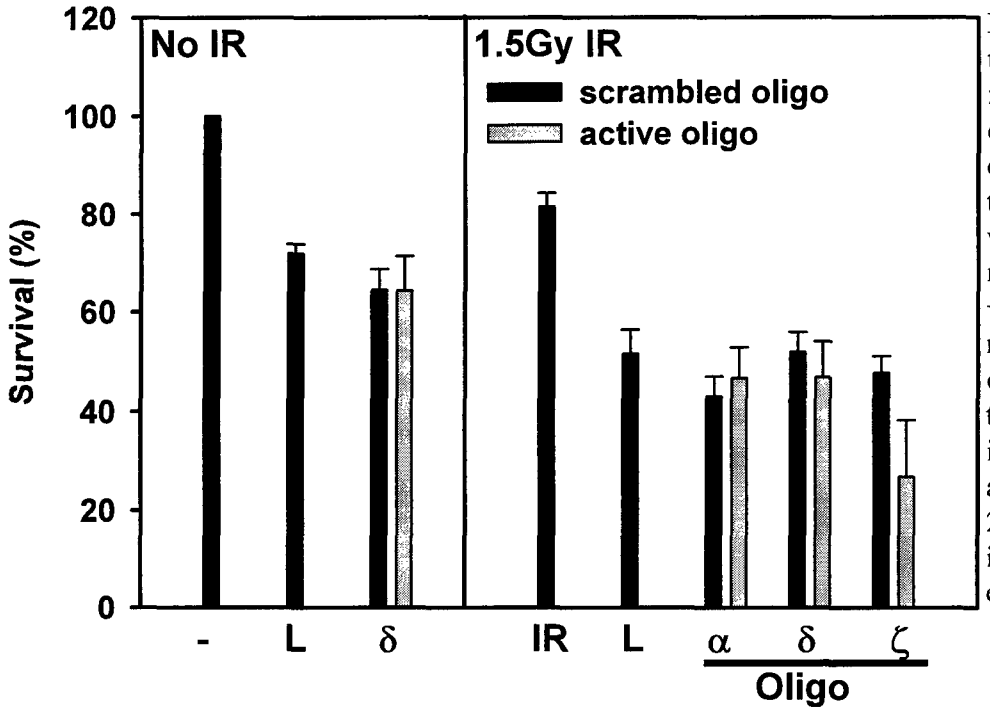
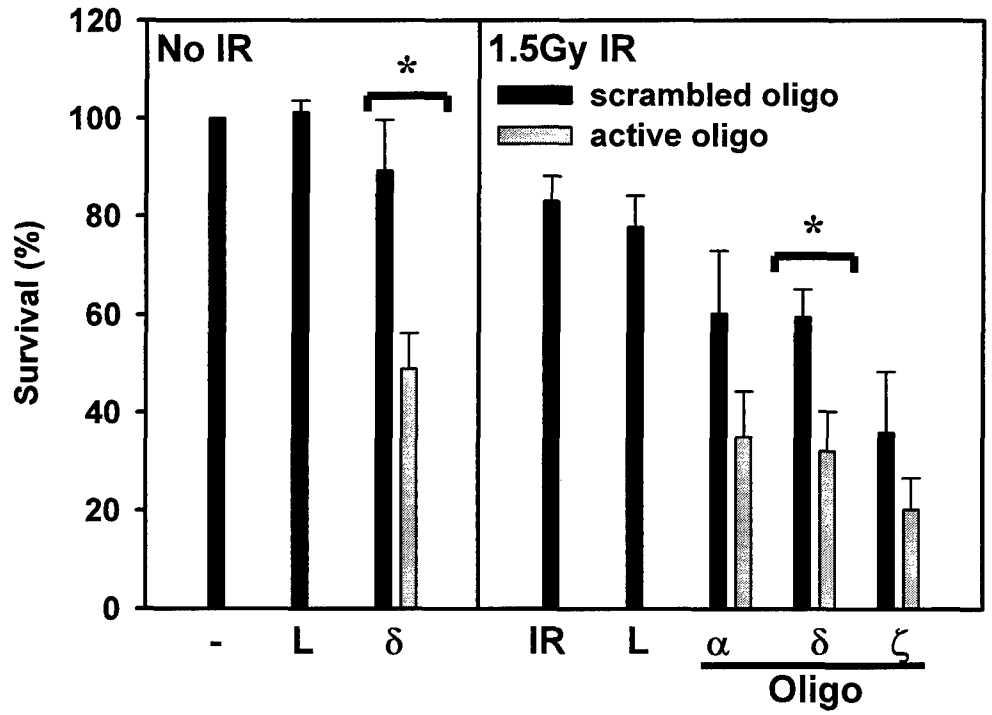



**Fig. 4. Cell survival in response to PKC oligo and low dose radiation treatment.** A, MCF-7 cells were treated with 200nM oligonucleotides plus liposome that target PKC α, δ and ζ. Controls were treated with nothing (-), radiation alone (IR), liposome (L) +/- IR, or liposome plus scrambled nucleotide versions of these oligonucleotides +/- IR. Post-transfection (48h), cells were irradiated with 1.5Gy, harvested and replated in 96-well plate at 2500 cells/well. After 7 days of incubation, cell survival was estimated using the MTS assay.




B, MDA-MB-231 cells were treated as in A but, with 100nM oligonucleotides. Cells were irradiated 24h post-transfection, harvested and replated at 250 cells/well. After 5 days incubation, cell survival was estimated using the MTS assay. Data are the mean of n=3±SE (A) or n=4±SE (B) independent experiments with 5 replicates/treatment. Survival of control cells (-) was set =100%. Statistically significant differences between treatment groups receiving PKC oligonucleotides versus nucleotide scrambled versions were determined by the Student t-test and are indicated (*$P<0.05$).

**Key Research Accomplishments:**

- PKC α, PKC δ and PKC ζ protein shown to be selectively depleted by PKC oligonucleotides (Task #3).

- Selective depletion of PKC δ and PKC ζ significantly reduce the survival of human breast cancer cells treated with high dose radiation (Task #4).

- Selective depletion of PKC δ impairs survival of non-irradiated and low dose irradiated MDA-MB-231 cells. MCF-7 cell survival was not reduced under either of these conditions (Task #4).

- Inhibition of the PKC δ enzyme with rottlerin impairs survival of non-irradiated, high or low dose irrradiated human breast cancer cells as measured by MTS and clonogenic survival assays (Task #4).

- Radiation survival curves for MCF-7 and MDA-MB-231 cells using the MTS and/or clongenic survival end-point assay(s) were established (Task #5).

- Laboratory is in the process of acquiring comet assay system to measure DNA damage (Task #6).

**Reportable Outcomes:**

Abstract and Poster Presentation: Susan G. Komen Breast Cancer Foundation Fourth Annual Conference: Reaching for the Cure... Making A Difference.
September 17-19, 2000, Washington, DC
"Combination Radiation and Antisense Oligonucleotide Gene Therapy for Breast Cancer."
M.A. McCracken and J.S. Strobl

Abstract and Poster Presentation: American Association for Cancer Research Annual Meeting March 24-28, 2001, New Orleans, LA
"Protein Kinase C Delta Involvement in Radiation-induced Mammary Tumor Cell Death."
M.A. McCracken, R.A. McKay, and J.S. Strobl, Abstract #3583
Proceedings of 92$^{nd}$ Annual American Association for Cancer Research, Vol. 42.

Awarded eligibility for attendance at the American Association for Cancer Research's "Pathobiology of Cancer Workshop" July 15-22, 2001, Keystone, CO.

Manuscript in progress: To be submitted to *Molecular Pharmacology* by July 31, 2001.
"Protein Kinase C Delta is a Pro-survival Factor in Human Breast Tumor Cell Lines"
M.A. McCracken, L.J. Miraglia, R.A. McKay, and J.S. Strobl

**Conclusions:**

The goal of this project is to increase radiation-induced breast cancer cell death through selective depletion of PKC isoforms. Western blot analysis showed that antisense oligonucleotides are a selective and efficient method for depleting specific PKC isoforms from human breast tumor cells. Protein extracts from cells treated with PKC α, PKC δ or PKC ζ oligonucleotides showed significant reduction in their respective PKC protein levels compared to treatment with the nucleotide scrambled versions of the oligonucleotides. Depletion of PKC δ and PKC ζ protein from MCF-7 and MDA-MB-231 cells prior to radiation (5.6Gy) with oligonucleotides corresponded with significanctly impaired survival as measused by MTS metabolism. However, pre-treatment with PKC α, PKC ε or PKC η oligonucleotides did not significantly reduce cell survival compared to treatment with nucleotide scrambled oligonucleotide controls. These results indicate that specific roles for individual PKC isoforms in the radiation survival of breast tumor cells do exist. The small molecule PKC δ inhibitor, rottlerin, produced results consistent with PKC δ inhibition by oligonucleotides. Rottlerin effectively reduced the survival of MCF-7 and MDA-MB-231 cell lines in the absence of radiation treatment or in the presence of low (1.5Gy) or high (5.2Gy) dose radiation treatment. To investigate the effect of PKC δ inhibition on cells exposed to a therapeutically relevant dose of radiation, cells were pre-treated with PKC δ oligonucleotide and then exposed to 1.5Gy radiation. The survival of MCF-7 cells was not impaired by this treatment. However, MDA-MB-231 cell survival was reduced 2-fold. These findings provide evidence that PKC δ and PKC ζ are pro-survival factors in mammary tumor cells. This work identifies PKC δ and PKC ζ as potential new molecular targets for breast cancer drug development and supports further investigation with PKC δ and PKC ζ antisense oligonucleotides.

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**Title Page**

# PKC δ is a Pro-survival Factor in Human Breast Tumor Cell Lines

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**Running Title Page**

# PKC δ is a Pro-survival Factor in Human Breast Tumor Cell Lines

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Text Pages: 24
Tables: 0
Figures: 6
References: 39
Abstract word count: 240
Introduction word count: 712
Discussion word count: 891

Nonstandard Abbreviations: IR, ionizing radiation; PKC, protein kinase C; MCF-7, Michigan Cancer Foundation; MDA-MB-231, M.D. Anderson metastatic breast; DAG, diacylglycerol; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κB; IKK, IκB kinase; DMEM, Dulbecco's modified Eagle's medium; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; FBS, fetal bovine serum; TPA, 12-O-tetradecanoylphorbol 13-acetate; ER, estrogen receptor; MDR, multi-drug resistance; ODC, ornithine decarboxlyase

**UNPUBLISHED DATA**

**Abstract**

Protein kinase C (PKC) promotes cell survival in response to ionizing radiation in a variety of experimental models including human carcinoma, human glioblastoma, and transformed mouse embryo fibroblast cell lines. We have introduced specific antisense oligonucleotides into human mammary tumor cell lines *in vitro* to analyze the role of individual PKC isoforms in radiation-induced cell death in breast cancer. MDA-MB-231 and MCF-7 cells treated with oligonucleotides directed against the PKC isoforms δ and ζ exhibited impaired survival in response to 5.6Gy gamma radiation as measured by mitochondrial metabolism of tetrazolium dye. PKC ε oligonucleotides had no affect on cell survival in either cell line while cells treated with PKC η oligonucleotides showed a somewhat enhanced cell survival that was significant in the MCF-7 line. The role of PKC δ in the breast tumor cell lines was of particular interest because contradictory reports exist in the literature regarding the role of PKC δ in cell survival and apoptosis. A comparison of the effects of the PKC δ antisense oligonucleotide and a nucleotide scrambled version of this nucleotide revealed only the antisense oligonucleotide decreased cell survival. The PKC δ antisense oligonucleotides decreased cell survival after exposure to low (1.5Gy) radiation doses, and in the absence of radiation insult. Furthermore, we found 3μM rottlerin, a selective PKC δ inhibitor, to reduce MCF-7 clonogenic survival by 78%. We conclude that PKC δ acts as a pro-survival factor in human breast tumor cells *in vitro*.

**UNPUBLISHED DATA**

## Introduction UNPUBLISHED DATA

The protein kinase C (PKC) family of serine/threonine kinases mediate intracellular responses to a variety of stimuli, including growth factors, hormones, and neurotransmitters. PKC is widely distributed in mammalian tissues. Some isoforms are expressed ubiquitously while the expression of other isoforms is restricted to specific tissues. Twelve PKC isoforms are distinguished on the basis of protein homology and cofactor utilization. PKC isoforms are divided into three subfamilies, classical (α, βI, βII, and λ), novel (δ, ε, η, θ, and μ), and atypical (ζ and ι/γ). Classical PKC isoforms posses a $Ca^{2+}$ binding domain and two cysteine-rich zinc fingers that are involved in diacylglycerol (DAG) binding. While the novel PKC isoforms contain the DAG binding sites, they lack the $Ca^{2+}$ binding domain and differ from the atypical isoforms, which require neither, $Ca^{2+}$ or DAG for activation. The PKC isoform profile determined in the human mammary epithelial cell line, MCF-7, includes PKC α, δ, ε, η, γ, ι, μ, and ζ isoforms. MDA-MB-231 human mammary epithelial cell lines express a PKC isoform profile very similar to MCF-7 cells with the exception that PKC α is highly expressed in MDA-MB-231 cells and weakly expressed in MCF-7 cells (Morse-Gaudio et al., 1998; Ways et al., 1995).

PKC participates in abnormal growth processes such as carcinogenesis, tumor progression, and inflammation (Goekjian and Jirousek, 1999). As the primary intracellular receptor for the tumor promoting phorbol esters, PKC plays a role in stimulating early events in early tumor formation. Proliferative signals converging on mitogen-activated protein kinase (MAPK), from G-protein coupled receptors and the receptor tyrosine kinases for epidermal- and platelet-derived growth factor are transduced through PKC (van Biesen et al., 1995) and could participate in tumor promotion. In addition, a role for PKC in the later stages of tumor development is

suggested by the observation that the metastastic capacity of tumor cells and PKC activity are positively correlated (Carey et al., 1999). Activation of PKC by 12-O-tetradecanoylphorbol 13-acetate (TPA) has been shown to increase extracellular matrix attachments, while reduction of these attachments has been observed in response to PKC inhibition (rev. Herbert, 1993). Overexpression of the PKC α isoform in MCF-7 cells resulted in reduced extracellular matrix attachments, by alterations in integrin heterodimer expression, facilitating metastatic growth (Carey et al., 1999).

PKC overexpression and increased PKC activity has been observed in human breast cancers compared with normal mammary tissue (O'Brian et al.,1989; Gordge et al., 1996). Furthermore, there is a correlation between elevated PKC protein levels and aggressive breast cancer phenotypes, such as those that lack estrogen receptors (ER) and exhibit multidrug resistance (MDR) (Borner et al., 1987; Lee et al., 1992). Relatively little is known about cell type specific functions of the individual isoforms in mammary epithelium, however overexpression of PKC α and PKC δ in mammary cells elicited responses that were clearly distinct from those in some other cell types. Overexpression of PKC α, stimulated MCF-7 breast tumor and C6 glioma cell growth (Ways et al.,1995; Baltuch et al., 1995), but overexpression of this same PKC isoform inhibited growth of bovine aortic endothelial cells and rat embryonic smooth muscle cells (Rosales et al., 1998; Wang et al., 1997). Additionally, PKC δ overexpression in rat mammary adenocarcinoma cells stimulated anchorage independent growth (Kiley et al., 1999a), but inhibited growth of cells of ovarian origin (CHO) as well as of mouse NIH3T3 cell fibroblasts (Watanabe et al., 1992; Mischak et al., 1993). A possible mechanism for inhibition of growth by PKC δ is through its reported function as a caspase 3 substrate during apoptosis (Cross et al., 2000). These observations indicate that generalizations about the role of particular PKC isoforms

without regard to cell context may not be valid. We conclude that cellular origins and the PKC isoform profiles are both important considerations in assigning cell type specific functional roles of PKC.

We have applied antisense oligonucleotide technology to explore isoform specific functions of PKC in the survival of human mammary carcinoma cell lines *in vitro*. We demonstrate that oligonucleotides can be used to selectively eliminate PKC δ from these cells and provide evidence that PKC δ functions as a pro-survival factor in human breast tumor cell lines. Further, our findings suggest that down-regulation of PKC δ may be a useful approach to radiosensitization in mammary tumors.

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## Materials and Methods UNPUBLISHED DATA

**Cell Culture.** MCF-7 (passage #39-50) and MDA-MB-231 human mammary tumor cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% FBS (Summit Biotechnology, Fort Collins, CO) and .04mg/ml gentamicin in a 7.5% $CO_2$, 37°C, humidified incubator. Cells were passaged weekly at 1:5 or 1:10 ratios, respectively. For clonogenic survival, MCF-7 cells were plated at $1x10^4$ cells/100mm$^2$ dish in 10ml DMEM/10% FBS and maintained for seven days in a 7.5% $CO_2$, 37°C, humidified incubator. To visualize, cells were stained with .5% crystal violet, 5% formalin, 50% ethanol, .85% NaCl. Colonies were scored using Nikon Eclipse TS100 microscope (Nikon, Japan) at 100X magnification with ≥10 cells =1 colony. For certain experiments, cells were treated with 1.5-9μM rottlerin (Sigma, St. Louis, MO) dissolved in dimethyl sulfoxide (DMSO).

**Antisense Oligonucleotides.** MCF-7 ($2.2x10^5$) or MDA-MB-231 ($1.1x10^5$) cells/35mm$^2$ dish were treated for 4-5 hours with 100-200nM α, δ, ε, η, or ζ active (ISIS #9606 or #119406, #13513, #13518, #17000, #13516), scrambled (ISIS #13009, #13514, #13517, #13520, #128585) or sense (ISIS #148051) PKC methoxy-ethoxy modified antisense oligonucleotide (Isis Pharmaceuticals, Carlsbad, CA) in 3μl Lipofectin (Life Technologies, Rockville, MD) transfection reagent/1mL Opti-Mem I reduced serum medium (Life Technologies). Transfection was stopped by medium exchange with DMEM/2% FBS.

**Radiation.** Cells were exposed to 1.5 to 5.6Gy doses of γ-ionizing radiation delivered with a Cesium$^{137}$ source in a Gammacell 40 (Atomic Energy of Canada Ltd., Ottawa) at 108.7 or

Gammacell 1000 (Atomic Energy of Canada Ltd.) at 730.0 rads/min under ambient temperature and atmospheric conditions. After radiation medium was replaced with DMEM/10%FBS.

**MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) Assay.** Metabolism of MTS was used as index of cell viability. MCF-7 or MDA-MB-231 cells were plated at 1500-2500 or 250 cells/well in a 96 well plate in 225μl DMEM/10% FBS. After plating (7 or 5 days), fresh growth medium (100ul DMEM/5% FBS + 20μl Cell Titer 96 (Promega, Madison, WI)) was added. Conversion of MTS reagent to formazan product was measured by absorbency at 490nm with Spectra Max 340pc plate reader (Molecular Devices, Sunnyvale, CA) after a 2 h incubation at 37°C. The MTS assay was linear under our assay conditions for 3 hours.

**Western Blotting.** MCF-7 ($7.0x10^5$) or MDA-MB-231 ($4.5x10^5$) cells/$100mm^2$ dish were rinsed one time with PBS. Total cellular proteins were collected by syringe in 100-150μl boiling lysis buffer (1%SDS, 10mM Tris, pH 7.4) and chilled on ice. Proteins were boiled for 5 minutes then centrifuged at 4°C, 13,000rpm in an Eppendorf Centrifuge 5415 C (Brinkmann Instruments Inc., Westbury, NY). Aprotinin (1mM), leupeptin (1mM), and phenylmethyl sulfonyl fluoride (100μM) were added to the supernatant. Protein concentrations were determined using the BCA assay (Pierce, Rockford, IL). Protein samples diluted in 1X sample buffer (.06M Tris-HCl, 10% glycerol, 2% SDS, .2% bromphenol blue, 2.5% 2-mercaptoethanol, pH 6.8)/1mM dithiothreitol and then resolved on 7.5% acrylamide gels at 100V were transferred to polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA) at 25V for 2 hours. Purified PKC α, δ, ε, η, and ζ proteins (#539674, #539673 Calbiochem, San Diego, CA) were used as standards. Membranes were blocked at 4°C, overnight in 3% non-fat dry milk/Tris buffered saline + .05% Tween20. Membranes were incubated with PKC α, δ, or ε mouse monoclonal antibody (mAb) (#P16520,

#P36520, #P14820, BD Transduction Laboratories, San Diego, CA), PKC ζ goat polyclonal (p) Ab, PKC η rabbit pAb or bcl-2 mouse mAb (SC #216-G, #215, #509, Santa Cruz, CA) for 3 hours 15 minutes (1:250 dilution for all primary Abs). Membranes were hybridized with a 1:1000 dilution of anti-mouse (SC #2005), 1:8000 of anti-rabbit (SC #2004), or a 1:10,000 dilution of anti-goat (SC #2020) HRP-conjugated secondary antibody for 30 minutes. Signals were visualized by Super Signal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and exposed to film. Signals were quantitated by FluorChem (Alpha Innotech, San Leandro, CA) spot densitometry using automatic background subtraction and normalized to a 200kDa protein band on the Gel Code Blue (Pierce) stained acrylamide gel.

**Statistical Analysis.** Statistically significant differences ($P<0.05$) were determined using the Student t-test. For multiple comparisons one-way ANOVA with Tukey's Multiple Comparison Test was used.

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## Results UNPUBLISHED DATA

**Radiation Survival Curves.** Cell survival following exposure to increasing doses of gamma radiation was measured using mitochondrial metabolism of MTS as an end-point. This assay is rapid, reproducible and suitable for screening purposes. Using the MTS assay, two human mammary tumor cell lines, MDA-MB-231 and MCF-7, used for these studies showed similar radiation survival curves. MCF-7 cell clonogenic survival measured using crystal violet staining and visual inspection of colonies was a more sensitive indicator of radiation-induced tumor cell damage. However, this method was not suitable for analysis of the MDA-MB-231 cells because these cells failed to form discrete colonies.

**Differential Expression of PKC Isoforms in MDA-MB-231 and MCF-7 Cells.** Individual PKC isoforms are differentially expressed within human mammary tumor cells. Western blot analysis of MDA-MB-231 and MCF-7 cell extracts was performed to examine the relative protein levels of PKC $\alpha$, PKC $\delta$, PKC $\varepsilon$, PKC $\eta$ and PKC $\zeta$ (Fig. 2). The major difference in the PKC expression profile between these two cell lines is the PKC $\alpha$ isoform expression. PKC $\alpha$ is present at higher levels in MDA-MB-231 cells than in MCF-7. MDA-MB-231 and MCF-7 cells also differed in their expression of the PKC $\eta$ isoform. While, MCF-7 cells contain two species of PKC $\eta$, a faster and slower migrating form, PKC $\eta$ was undetectable in MDA-MB-231 cells. The expression of PKC $\delta$, PKC $\varepsilon$ and PKC $\zeta$ were similar between the two cell lines, with slightly stronger signals detected in MCF-7 cell extracts.

**PKC $\delta$ and $\zeta$ Oligonucleotides Decrease Survival in Human Breast Tumor Cells.** PKC gene and protein expression are induced following ionizing radiation (Woloschak et al., 1990; Kim et al., 1992). PKC inhibition radiosensitizes human squamous carcinoma, human colon adenocarcinoma, transformed mouse embryo fibroblast, and human glioblastoma cell lines

(Hallahan et al., 1992; Zaugg et al., 2001; Rocha et al., 2000; Begemann et al., 1998). Our goal was to examine the roles of specific PKC isoforms on cell survival in response to radiation insult by 5.6Gy. For our initial screen oligonucleotides that targeted individual PKC isoforms representing the classical (α), novel (δ, ε and η), and atypical (ζ) PKC families were introduced into two human breast tumor cell lines using liposomes. Treatment of MDA-MB-231 cells with PKC δ oligonucleotide decreased cell survival ($P<0.05$) in the irradiated cells by 44% compared with cells that were treated with the nucleotide scrambled version of this oligonucleotide (Fig. 3A). Liposome treatment alone or liposome treatment plus the nucleotide scrambled version of the PKC δ oligonucleotide had no effect on MDA-MB-231 survival indicating that the oligonucleotide sequence was critical to the decreased survival. Cell survival in response to radiation was also significantly decreased in the MDA-MB-231 cells treated with PKC ζ oligonucleotide compared with the cells that were treated with the nucleotide scrambled version of the PKC ζ ($P<0.05$). In this pair of oligonucleotides, one can see that the nucleotide scrambled version reduced cell survival compared to liposome treatment alone indicating that there was some sequence non-specific effect upon cell survival. However, in response to radiation the PKC ζ oligonucleotide resulted in a marked overall decrease in cell survival from 91% (liposome treatment alone) to 32% (liposome plus PKC ζ oligonucleotide). Oligonucleotide treatment alone was insufficient to decrease MDA-MB-231 cell survival in response to 5.6Gy of gamma radiation. PKC η oligonucleotide and its nucleotide scrambled version had no effect upon MDA-MB-231 cell survival. These data provide additional support for our hypothesis that PKC isoform specific influences upon breast tumor cell survival following ionizing radiation exist and suggest the effects of PKC δ and PKC ζ oligonucleotides upon survival of irradiated MDA-MB-231 cells are quite specific. In MCF-7 cells (Fig. 3B), liposome treatment alone reduced survival of

irradiated cells by approximately 50%, and the magnitude of the liposome effect exceeded that of oligonucleotide treatment in affecting cell survival after gamma irradiation. Nevertheless, PKC δ and PCK ζ oligonucleotides significantly decreased the survival of MCF-7 cells after radiation treatment, thus providing confirmatory evidence for the importance of these two PKC isoforms in the radiation survival of human breast tumor cell lines. The results of experiments using the PKC α oligonucleotides and the PKC ε oligonucleotides were not conclusive. In cells treated with either the isoform specific oligonucleotide or the nucleotide scrambled version of the specific oligonucleotide, cell survival decreased following irradiation. Further experimentation that uses alternative versions of the nucleotide scrambled oligonucleotides is needed to clarify the role of PKC α and PKC ε in the survival of gamma irradiated cells. The non-specific effects of oligonucleotides can only be distinguished from isoform specific actions when a nucleotide scrambled version of the oligonucleotide has no effect upon cell survival.

**PKC δ Oligonucleotides Specifically Reduce PKC δ Protein Levels.** To further analyze the role of PKC δ oligonucleotide in breast tumor cell survival post-irradiation, we tested whether PKC δ protein levels were decreased in cells treated with oligonucleotide that targeted PKC δ. Whole cell extracts were prepared from MCF-7 and MDA-MB-231 cells either 52 h or 72 h after transfection with PKC δ oligonucleotide. No PKC δ protein was detected in either cell line after 72 h (Fig. 4). Untreated cells, cells treated with lipofectin alone or the inactive nucleotide scrambled version of the PKC δ oligonucleotide showed constant levels of PKC δ at 52 and 72 h. We conclude that the PKC δ oligonucleotide transfection protocol, but not the control conditions used in the radiation survival experiments, depleted human breast tumor cell lines of PKC δ protein. To test whether the effect of PKC δ oligonucleotide was selective for the δ isoform of

protein kinase C, protein levels of PKC ε and PKC ζ were assayed in the MCF-7 cell extracts (Fig. 4). There was no effect of the PKC δ oligonucleotide on levels of either PKC ε or PKC ζ. Bcl-2 is an anti-apoptotic protein that is highly expressed in human breast tumor cells (Eissa et al., 1999). We tested whether the effect of PKC δ oligonucleotide could be manifested as a decrease in bcl-2 protein levels by immunoblot analysis. Figure 4 shows that the levels of bcl-2 protein are constant in MCF-7 and MDA-MB-231 cells treated with the PKC δ oligonucleotide. The decreased survival of irradiated cells following depletion of PKC δ protein with PKC δ oligonucleotide can not be attributed to a decrease in bcl-2 protein. We hypothesize that PKC δ protein is a survival factor in these two human breast tumor cell lines, and that oligonucleotide depletion of PKC δ decreases cell survival in response to gamma radiation.

**Rottlerin Reduces Cell and Clonogenic Survival.** To test the hypothesis that PKC δ is a survival factor in breast tumor cell lines, we performed clonogenic survival assays with MCF-7 cells following exposure to rottlerin, a PKC δ selective PKC inhibitor. Clonogenic survival assays are a sensitive measure of drug activity, and the MCF-7 cells were highly responsive to rottlerin under these conditions. Clonogenic survival of MCF-7 cells exposed to 3μM rotterin for 7 days, was reduced by 78% compared to control cells exposed to the solvent alone (Fig. 5A). The published $IC_{50}$ value for rottlerin inhibition of purified PKC δ is 3-6μM (Gschwendt et al., 1994). These results support the hypothesis that PKC δ is a survival factor in breast tumor cells. We also tested whether rottlerin would decrease survival of MCF-7 and MDA-MB-231 cells following gamma radiation (Fig. 5B and C). MCF-7 cells were exposed to either 1.5 or 5.2Gy of gamma radiation. MDA-MB-231 cells were tested only at the 5.2Gy radiation dose. After radiation, the cells were harvested and plated into 96-well dishes containing medium with varying concentrations of rottlerin. MCF-7 cell radiation survival measured by the MTS assay

was reduced by treatment with 3μM rottlerin. MDA-MB-231 cells were even more sensitive to 3μM rottlerin, in the presence or absence of radiation (Fig. 5C) than MCF-7 cells.

**PKC δ Oligonucleotide Decreased Breast Tumor Cell Survival in Response to 1.5Gy Gamma Radiation.** Breast cancer radiation therapy is administered in the range of 1-2Gy/treatment. To determine whether PKC δ oligonucleotide would decrease breast tumor cell survival *in vitro* in this radiation dose range, cells were exposed to 1.5Gy radiation 17 h after transfection with oligonucleotides (Fig. 6). Control, non-irradiated cells were treated in parallel with PKC δ oligonucleotide or its nucleotide scrambled version. When MCF-7 cells were pre-treated with the PKC δ oligonucleotide, there was no significant difference in the cell or clonogenic survival in comparison to treatment with the scrambled oligonucleotide (data not shown). Consistent with earlier results shown in Figure 3, a 40% decrease in cell survival over 5 days was observed in non-irradiated MDA-MB-231 cells treated with the PKC δ compared with the nucleotide scrambled oligonucleotide (Fig. 6). Survival of the MDA-MB-231 cells exposed to the low dose of gamma radiation (1.5Gy) alone was decreased approximately 20% compared to non-irradiated controls. Pre-treatment of the cells with the PKC δ oligonucleotide caused cell survival to decrease by approximately 70%. Cell survival in irradiated cells pre-treated with the PKC δ oligonucleotide was statistically signifcantly reduced compared with the cells that were pre-treated with the nucleotide scrambled version of the oligonucleotide. We conclude that PKC δ oligonucleotide is a radiosensitizer in MDA-MB-231 human breast tumor cells.

**UNPUBLISHED DATA**

**Discussion UNPUBLISHED DATA**

In the present study, we showed that selective depletion of the PKC δ isoform decreased human mammary tumor cell survival. Our findings suggest that PKC δ positively regulates survival in breast cancer cells. Additional studies showed that the atypical protein kinase C, PKC ζ, also functions as a pro-survival factor in breast cancer. In contrast, PKC η may be growth suppressive because PKC η antisense oligonucleotide treatment improved MCF-7 cell survival. Gamma radiation was used to challenge survival of the breast tumor cells in these studies because the results of earlier experiments with the PKC inhibitors, staurosporine, sangivamycin and H7 provided evidence that PKC activation was radioprotective (Begemann et al., 1998; Hallahan et al., 1992; Zhang et al., 1993). PKC activation opposes the pro-apoptotic effects of radiation-induced ceramide generation (Haimovitz-Friedman et al., 1994), but the mechanism(s) for this response and the roles of the individual PKC isoforms are not well defined. This report begins to clarify the role of individual PKC isoforms that mediate survival in breast tumor cells in response to gamma radiation.

The two main approaches to isoform selective PKC inhibition are site selective enzyme inhibition or suppression of the mRNA levels. Oligonucleotides that target RNA are highly specific PKC isoform antagonists, and may be easier and less expensive to synthesize than traditional small molecule inhibitors targeted to the enzyme (Glazer, 1998). A few small molecule inhibitors offer PKC isoform specific inhibition. The small molecule inhibitor, rottlerin, is selective for the PKC δ isoform ($IC_{50}$ 3-6μM) but also inhibits calmodulin kinase III ($IC_{50}$=5.3μM) (Way et al., 2000). Our approach to elucidate the role of particular PKC isoforms involved in mammary tumor cell survival following radiation has been to use antisense oligonucleotides and where possible, small molecule inhibitors.

PKC δ is a novel PKC. This $Ca^{2+}$-independent isoform has been proposed to both regulate and serve as a substrate for caspases (Basu and Akkaraju,1999). Specifically, PKC δ is activated by caspase 3 during apoptosis in leukemia cells resulting in phosphorylation of lamin B (Cross et al., 2000), an event known to precede nuclear lamina disassembly during apoptosis. In response to TPA, relocalization of cytoplasmic PKC δ to the mitochondria of MCF-7 cells, preceding cytochrome c release has been observed (Majumbder et al., 2000). These findings support a pro-apoptotic role for PKC δ.

In contrast, growth promoting activities of PKC δ have also been observed in mammary tumor cells. PKC δ involvement in cytoskeleton-dependent processes is evident in MTLn3 mammary tumor cells. Expression of the inhibitory PKC δ regulatory domain (RD δ) inhibited growth in soft agar, cell motility and attachment (Kiley et al.,1999). Furthermore, in highly metastatic mammary tumor cells, PKC δ and ζ protein and mRNA are significantly increased relative to the less metastatic tumor cells (Kiley et al., 1999a; Kiley et al., 1996).

Ornithine decarboxylase (ODC) is an enzyme essential to cell proliferation. ODC is capable of inducing cell transformation when overexpressed and is frequently highly expressed in tumor cells. Dominant negative PKC δ expression in murine papilloma cells attenuates induction of ODC in response to oxidative damage (Otieno and Kensler, 2000). Thus, PKC δ might act to promote cell growth by facilitating the expression of ODC.

PKC ζ activates the MAPK signal transduction pathway in a Ras-independent manner (van der Hoeven et al., 2000; Berra et al., 1995) and activates nuclear factor κB (NFκB) by phosphorylation of IκB kinase (IKK) (Lallena et al.,1999). Both of these activities of PKC ζ might contribute to cell survival in radiation stressed cells and account for the decrease we

observed in cell survival of MCF-7 and MDA-MB-231 mammary tumor cells treated with PKC ζ antisense oligonucleotides.

In MCF-7 cells, treatment with the PKC η antisense oligonucleotide increased survival. The PKC η oligonucleotide had no effect on MDA-MB-231 cell survival. The absence of detectable immunoreactive PKC η in MDA-MB-231 cells is a logical explanation for the failure of PKC η oligonucleotide to affect cell survival. It is also interesting to note that MDA-MB-231 cells have high PKC α and no immunodectable PKC η (Fig. 2), because down regulation of PKC η has been reported to occur in breast cancer cells that overexpress PKC α (Ways et al., 1995). These preliminary observations suggest that high levels of PKC α expression and low immunodectable levels of PKC η may be correlated with a rapidly growing, aggressive breast tumor phenotype. The data lead us to hypothesize that PKC η elimination may increase survival.

PKC α is a molecular target which shows promise clinically for development of new anti-cancer therapeutic agents. The $Ca^{2+}$-dependent PKC α isoform has been shown to be involved with the acquisition of multi-drug resistant (Yu et al., 1991) and ER negative breast cancer cell phenotypes. Stable expression of PKC α in MCF-7 cells results in increased proliferation and anchorage-independent growth, as well as in a reduction of ER expression and estrogen mediated gene expression (Ways et al., 1995). Although, our studies with the PKC α isoform were inconclusive and will require further investigation, inhibition of PKC α by ISIS 3521 antisense oligonucleotide has shown anti-tumor activity in phase I and II clinical trials in solid tumors (Nemunaitis et al.,1999). ISIS 3521 is now undergoing phase III clinical trials. Our results identify PKC δ as a potential new molecular target for breast cancer drug development and support further investigation of ISIS 13513 as a therapeutic agent. **UNPUBLISHED DATA**

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## UNPUBLILSHED DATA

**Figure 1.** Radiation survival curves of MDA-MB-231 and MCF-7 cell lines. Cells were treated with 0-9.5Gy doses of IR. Following IR exposure (20 h) cells were harvested and replated at cloning cell densities in 96-well plates or $100mm^2$ dishes. After 5 or 7 days of incubation cell survival was determined by MTS (circles) and clonogenic survival (triangle) assays. Survival of control cells was set =100%. The efficiency of clonogenic survival in control cells was 11%. Data represent the mean of n=3 ± SE (MTS data) or n=1 (clonogenic survival) independent experiments.

**Figure 2.** PKC protein levels in MDA-MB-231 and MCF-7 cells. Extracts were prepared from MDA-MB-231 ($1x10^6$) or MCF-7 ($7.5x10^5$) cells/$100mm^2$ dish 24 h after plating. Proteins (40μg/lane- α, ε, η, ζ and 62μg/lane- δ) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC α, PKC δ, PKC ε, PKC η and PKC ζ. The PKC standards (STD) included purified PKC α (15ng/lane), PKC δ (15ng/lane), PKC ε (5ng/lane), PKC η (3ng/lane) and PKC ζ (40ng/lane). Data shown are typical of n=2 experiments.

**Figure 3.** Cell survival in response to gamma radiation +/- PKC oligonucleotide treatment. A, MDA-MD-231 cells were treated with 100nM oligonucleotides plus liposome that target PKC α, δ, ε, η and ζ (gray bars). Controls were treated with radiation alone (IR), radiation plus liposome (L), or radiation plus liposome plus scrambled nucleotide versions of these oligonucleotides (black bars). Post-transfection (24 h), cells were irradiated with 5.6Gy of gamma irradiation, harvested and replated in 96-well plates at a cloning density of 250 cells/well. After 5 days of incubation, cell survival was estimated using the MTS assay. B, MCF-7 cells were treated with

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200nM oligonucleotides that target PKC in an isoform-specific manner. Cells were irradiated with 5.6Gy gamma radiation 48 h post-transfection, harvested and replated in 96-well plates at a cloning density of 2500 cells/well. After 7 days of incubation, cell survival was estimated using the MTS assay. Data are the mean of n=4 ± SE (A) or n=3 ± SE (B) independent experiments performed with 5 replicates/treatment. Survival of cells treated with radiation alone was set =100%. Statisitically significant differences between treatment groups receiving PKC oligonucleotides versus nucleotide scrambled sequence PKC oligonucleotides are indicated (*P<0.05).

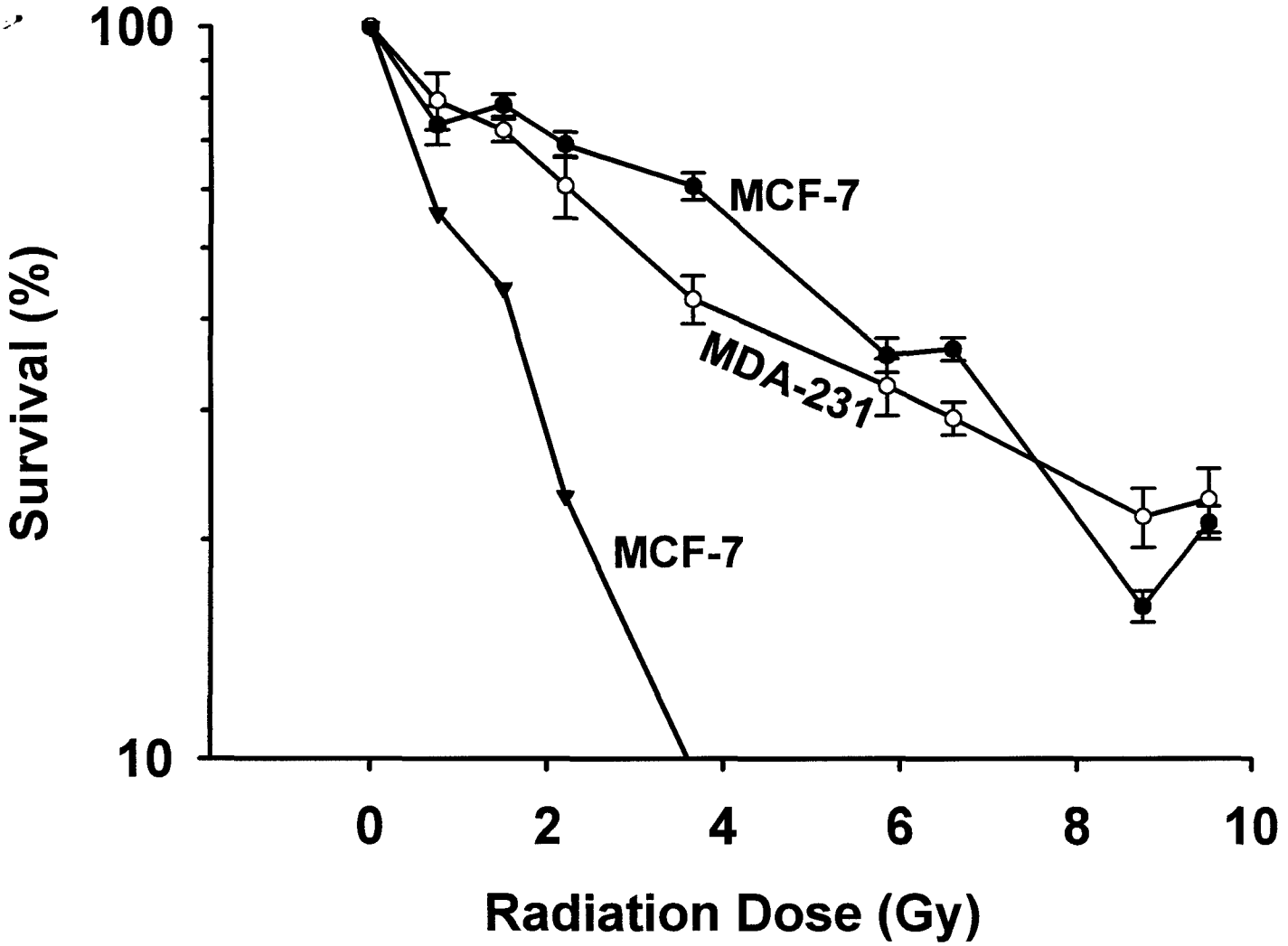
**Figure 4.** Immunoblot analysis of PKC δ oligonucleotide treated cells. A, Extracts were prepared from cells 52-72 h after treatment with lipofectin alone (L), PKC δ oligonucleotide (PKC δ), the nucleotide scrambled version (PKC δscr), or no treatment. Proteins (20μg/lane) were resolved on 7.5% polyacrylamide gels, transferred to filters and probed with antibodies to PKC δ, PKC ε, PKC ζ and bcl-2. The PKC standards (STD) included purified PKC δ (15ng/lane), PKC ε (5ng/lane) and PKC ζ (25ng/lane). Results are typical of three independent experiments.

**Figure 5.** Effect of rottlerin on cell survival. A, MCF-7 cells ($1.0x10^4/100mm^2$ dish) were treated with indicated concentrations of rottlerin. Cells were left undisturbed for 7 days then plates were stained with crystal violet to visualize colonies. The cloning efficiency for control cells was 11.0%. The clonogenic survival was determined for n=3 ± SE independent experiments with control clonogenic survival set =100%. Statistically significant differences between control and treatment groups are indicated (*P<0.05). B, MCF-7 cells ($3.0x10^5/35mm^2$ dish) were treated

with 1.5 or 5.2Gy IR, harvested, replated at a cloning density of 2500 cells/well, and treated with indicated concentrations of rottlerin. Cells were grown for 7 days and cell survival was determined by MTS assay. C, MDA-MB-231 cells ($2.0x10^5/35mm^2$ dish) were treated as in B but with 5.2Gy IR, replated at a cloning density of 250 cells/well, and grown for 5 days. Data are the mean of n=3 ± SE independent experiments performed with 5 replicates/treatment. Cell survival of control cells was set =100% (B and C).

**Figure 6.** PKC δ oligonucleotide sensitizes human breast tumor cells to low dose gamma radiation. MDA-MB-231 cells were transfected with PKC δ oligonucleotides and 17 h later irradiated with 1.5Gy gamma radiation, harvested and replated (250 cells/well) in 96 well-plates. MTS activity was measured after 5 days as an index of survival. Data are the mean n=4 ± SE independent experiments performed with 5 replicates/treatment. Statistically significant differences between the oligonucleotide treatments are indicated (*P <0.05).

**Figure 1. UNPUBLISHED DATA**



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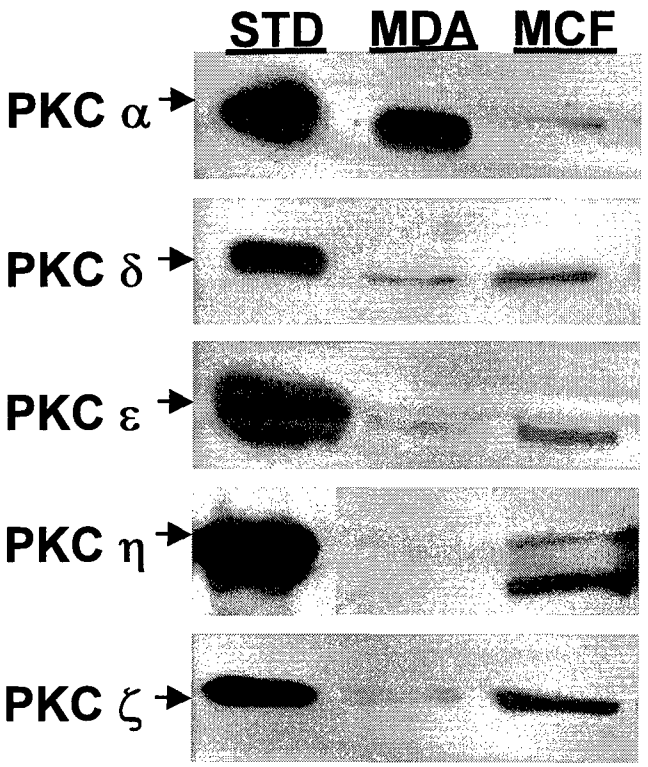

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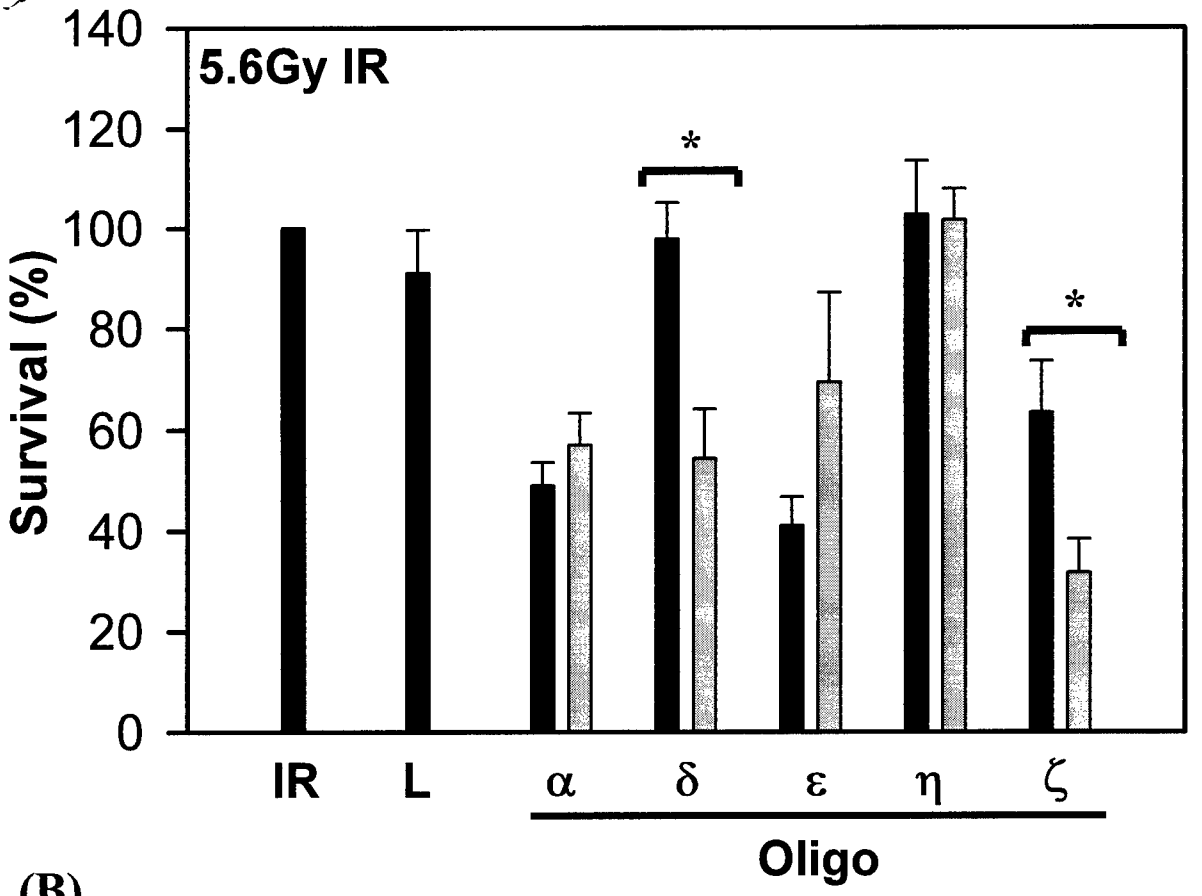


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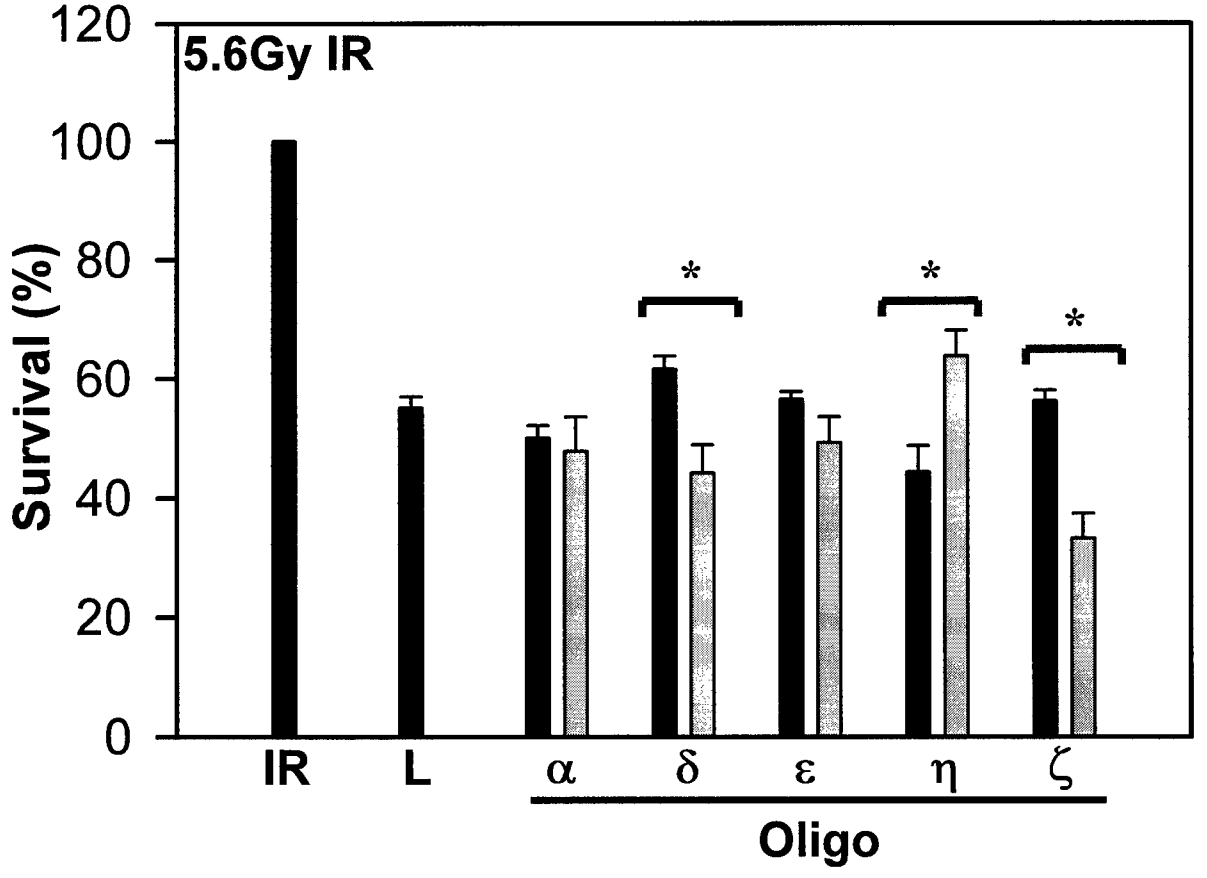

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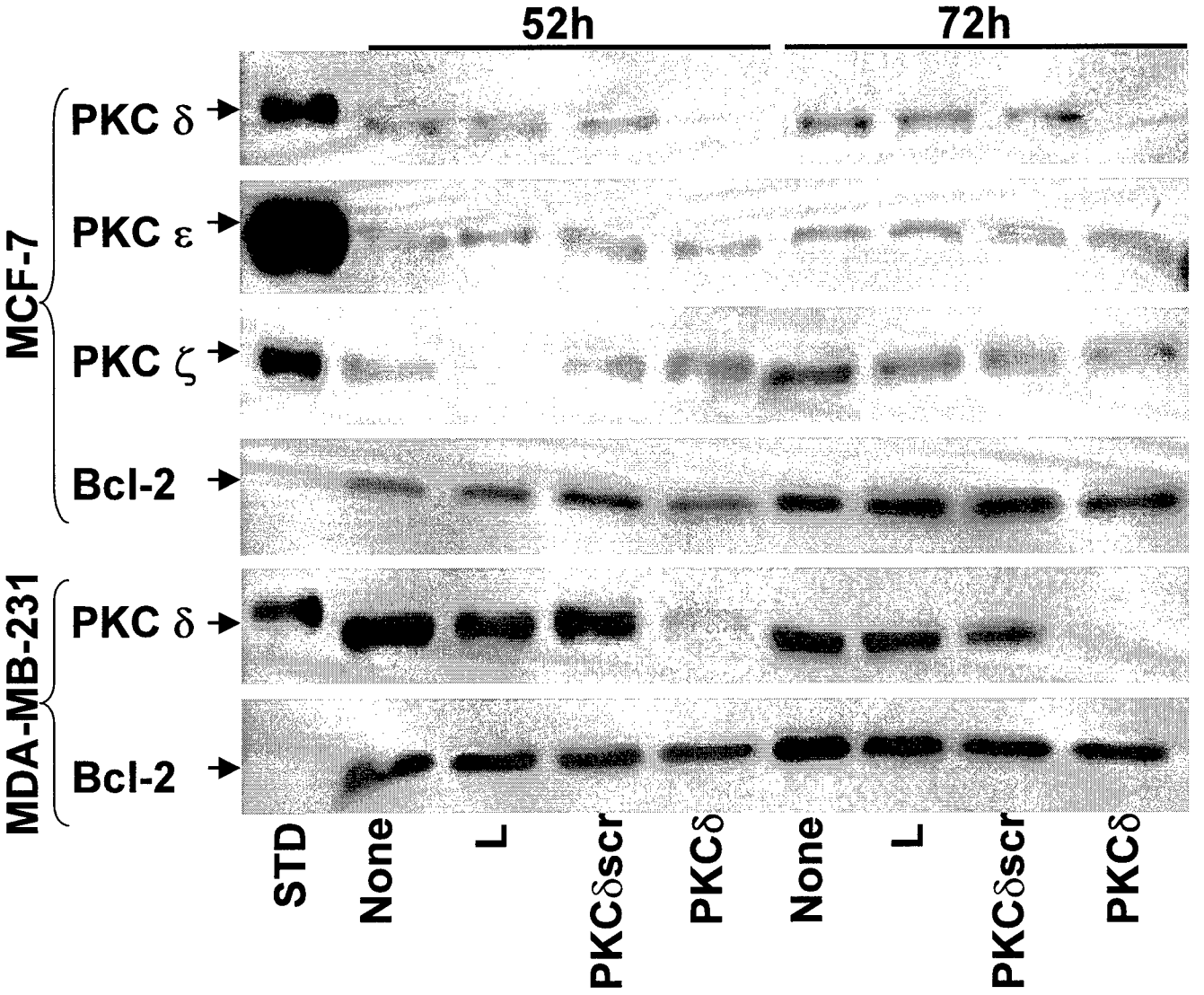
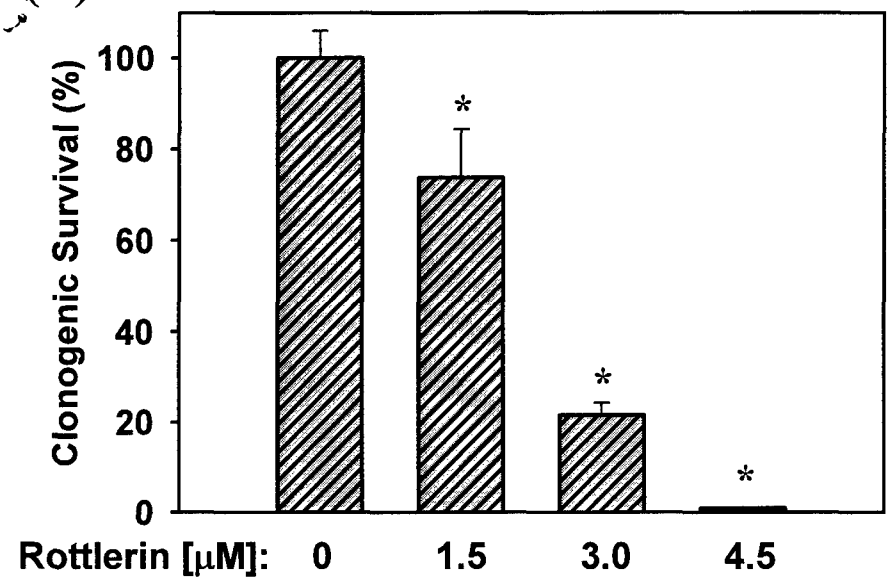


(A) **Figure 5. UNPUBLISHED DATA**

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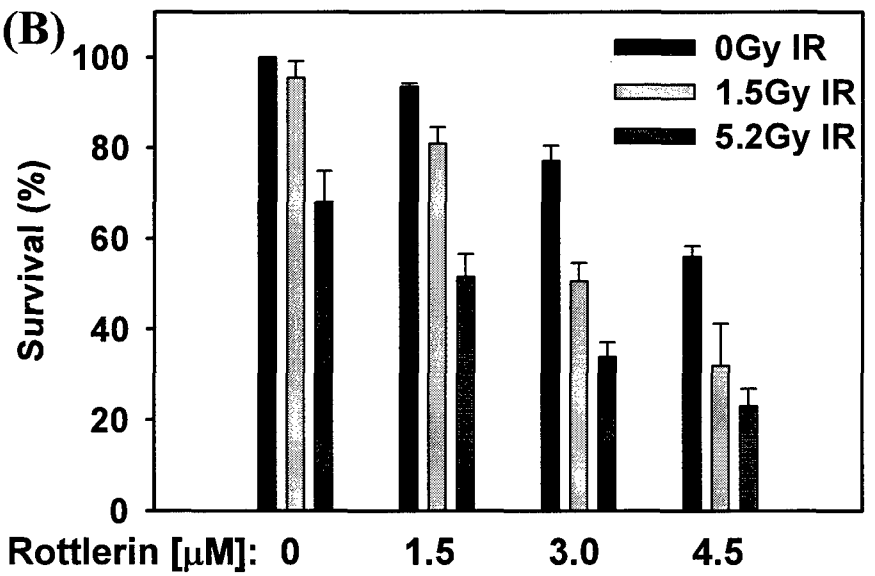


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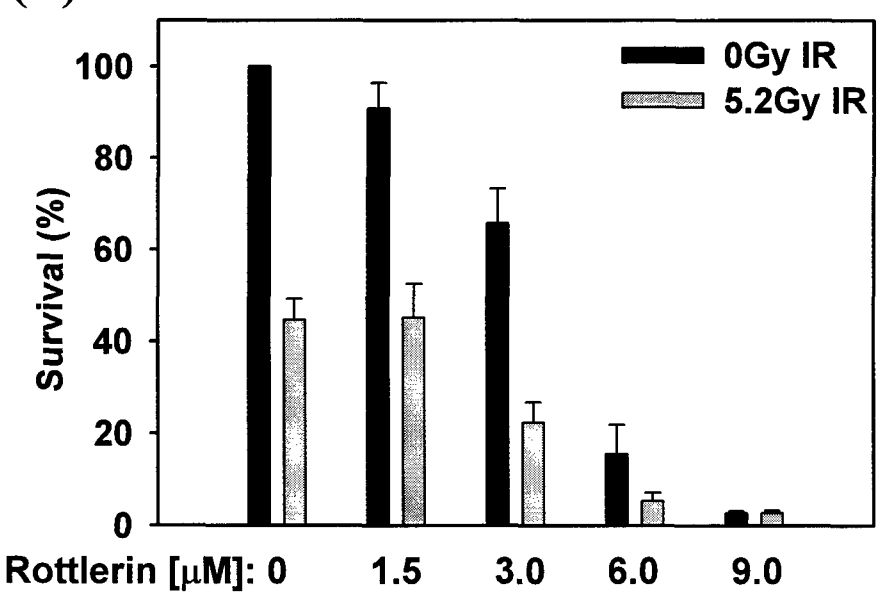

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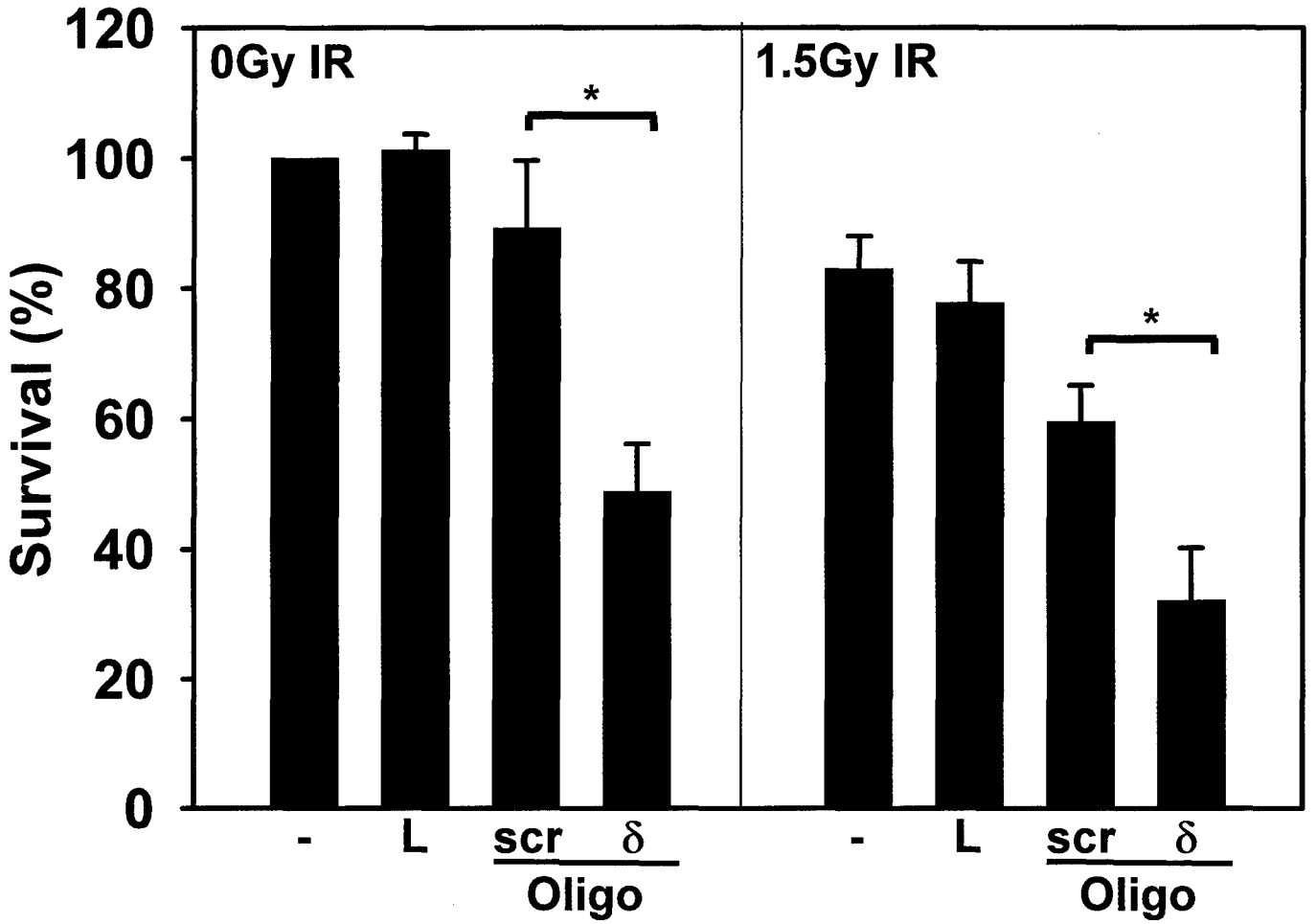

**Footnotes**

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Susan G. Komen Breast Cancer Foundation Grant # 993249

M.A. McCracken, R.A. McKay, and J.S. Strobl (2001) Protein Kinase C Delta Involvement in Radiation-induced Mammary Tumor Cell Death. *Proceedings of 92nd Annual American Association for Cancer Research* **42:**666, Abstract #3583.

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REPLY TO
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MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

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